

REMARKS / ARGUMENTS

The amendment to limit the proliferative disease to a solid tumor disease in claim 1 and other claims is supported by the specification, particularly at page 2, second paragraph.

Applicant believes that the numbering of the claims has been corrected. Accordingly, withdrawal of the objection is requested.

Applicant submits that the rejection of claim 8 under 35 USC 101 is rendered moot by the claim's cancellation.

Applicant submits that the amendments to claim 11 overcome the rejection of it under 35 USC 112, second paragraph. Therefore, withdrawal of this rejection is requested.

Claims 1-8 and 11 were rejected under 35 USC 112, first paragraph. Since the limitations of former claim 10, which was not included in the rejection, are now included in all of the pending claims, Applicant submits that this rejection is overcome by the claims here presented. Therefore, Applicants request withdrawal of the rejection.

Claims 1-9 and 10 (now claims 1-9) were rejected under 35 USC 102(e) as anticipated by Vite et al. Applicants request reconsideration and withdrawal of this rejection for the reasons that follow.

The Examiner cites the disclosure Vite et al at column 1, lines 14-30, where the structures of epothilones A and B are disclosed, and column 6, lines 18-20, where the reference discloses that the compounds of the invention can be used in combination with radiation. However, such a disclosure does not anticipate the presently claimed invention.

It is clear that Vite et al is concerned with compounds defined by the formula disclosed from column 1, line 38, to column 2, line 54, (formula V); and that epothilones A and B are disclosed as the prior art. It is also clear that the disclosure at column 6, line 18-41, relates to potential combination therapies that include "compounds of the invention" and an additional

therapeutic agent, and does not relate to combinations with the compounds defined as in present claims 2 and 3. Therefore, Vite et al does not even suggest the inventions defined by present claims 2 and 3, which are limited to combinations of radiation and compounds that are clearly excluded from the scope of Vite et al's generic formula V.

Moreover, Vite et al's disclosure at column 6, line 18-41, discloses that all of the thousands or millions of compounds included within the scope of the reference's formula V could be combined with any and all known anti-cancer and cytotoxic agents and treatments. However, such a disclosure does not anticipate each and every possible combination. One must look to the specific teachings of the reference to determine whether any of the potential combinations is anticipated. However, since the reference does not appear to contain any additional disclosure relating to combinations of the disclosed compounds with radiation, none of the combinations included within the scope of the present claims is anticipated by the reference.

In addition, since preference is taught for combinations that include a compound of formula V and the additional treatments mentioned from column 6, lines 27-41, it is readily inferred that radiation was mentioned only to be clear. Such a disclosure would not lead one of skill in the art to have a reasonable expectation of success when combining any of the disclosed compounds with radiation. Therefore, the invention of present claim 1 is not even obvious over Vite et al. Since the reference clearly teaches a preference for compounds different from those included within present claims 2 and 3, if it has any teaching relating to those compounds, it should be construed as teaching away from present claims 2 and 3.

Enclosed herewith are two journal articles: B. Hofstetter et al, Clinical Cancer Research, Vol. 11, 1588-1596 (2005) and C. Bley et al, Clinical cancer Research, Vol. 15(4), 1335-1342 (2009). The 2005 article provides evidence of a multilayered synergistic response on the cellular and tumor tissue levels induced by the combination of radiation and patupilone (aka epothilone B). The 2009 article discusses experiments relating determining the mechanism of combined epothilone B/radiation treatment. Applicants assert that the experiments discussed in these articles support the patentability of the present claims.

Applicant requests withdrawal of the rejection under 35 USC 102(e) for the reasons discussed above.

Claim 11 (now claim 10) was rejected under 35 USC 102(e) as anticipated by Bandyopadhyay et al. Applicant requests reconsideration of this rejection for the reasons that follow.

Applicants will refer to the rejected claim as claim 10 in view of the present amendment.

The reference is clearly concerned with oral administration of epothilones and discloses that the bioavailability of orally administered epothilones is enhanced if an acid neutralizing buffer is also administered with the oral epothilone. At pages 6 and 7, the reference teaches kits that comprise an orally administrable formulation of an epothilone and an orally administrable solution of an acid neutralizing buffer. This kit is clearly concerned with use of the buffer to improve oral bioavailability of the epothilone.

Although the kit would presumably contain an instruction on how to use it, Bandyopadhyay et al does not disclose or suggest such a kit which includes an instruction to use the epothilone in combination with ionizing radiation for the treatment of a solid tumor disease. However, such instructions are a critical limitation of present claim 10. Therefore, the reference does not identically disclose the invention of the rejected claim. For this reason, an anticipation rejection is improper. Accordingly, Applicants request withdrawal of the rejection of claim 10 under 35 USC 102(e).

Entry of this amendment and reconsideration and allowance of the claims are respectfully requested.

Respectfully submitted,



George R. Dohmann
Attorney for Applicant
Reg. No. 33,593

Novartis Pharmaceuticals Corp.
Patents Pharma
One Health Plaza, Building 104
East Hanover, NJ 07936-1080
(862) 778-7824
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C. Bley et al, Clinical cancer Research, Vol. 15(4), 1335-1342 (2009)

Patupilone Acts as Radiosensitizing Agent in Multidrug-Resistant Cancer Cells *In vitro* and *In vivo*

Barbara Hofstetter,^{1,2} Van Vuong,¹
 Angela Broggini-Tenzer,¹ Stephan Bodis,¹
 Ilja F. Ciernik,¹ Doriano Fabbro,³
 Markus Wartmann,³ Gerd Folkers,² and
 Martin Prusky¹

¹Department of Radiation Oncology, University Hospital Zurich;
²Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology, Zurich, Switzerland; and ³Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland

ABSTRACT

Interference with microtubule function is a promising antitumoral concept. Paclitaxel is a clinically validated tubulin-targeting agent; however, treatment with paclitaxel is often limited by taxane-related toxicities and is ineffective in tumors with multidrug-resistant cells. Patupilone (EPO906, epothilone B) is a novel non-taxane-related microtubule-stabilizing natural compound that retains full activity in multidrug-resistant tumors and is clinically less toxic than paclitaxel. Here we have investigated the effect of combined treatment with ionizing radiation and patupilone or paclitaxel in the P-glycoprotein-overexpressing, p53-mutated human colon adenocarcinoma cell line SW480 and in murine, genetically defined *E1A/ras*-transformed paclitaxel-sensitive embryo fibroblasts. Patupilone and paclitaxel alone and in combination with ionizing radiation reduced the proliferative activity of the *E1A/ras*-transformed cell line with similar potency in the sub and low nanomolar range. SW480 cells were only sensitive to patupilone, and combined treatment with low-dose patupilone (0.1 nmol/L) followed by clinically relevant doses of ionizing radiation (2 and 5 Gy) resulted in a supra-additive cytotoxic effect. Inhibition of the drug efflux protein P-glycoprotein with verapamil resensitized SW480 cells to treatment with low doses of paclitaxel alone and in combination with IR. In tumor xenografts derived from SW480 cells a minimal treatment regimen with patupilone and fractionated irradiation (1 × 2 mg/kg plus 4 × 3 Gy) resulted in an at least additive tumor response with extended tumor growth arrest. Analysis by flow cytometry *in vitro*

revealed an apoptosis- and G₂-M-independent mode of radiosensitization by patupilone. Interestingly though, a transient accumulation of cells in S phase was observed on combined treatment. Overall, patupilone might be a promising alternative in paclitaxel-resistant, P-glycoprotein-overexpressing tumors for a combined treatment regimen using ionizing radiation and a microtubule inhibitor.

INTRODUCTION

Interference with microtubule function represents a clinically validated mechanistic concept for anticancer drugs. Microtubule-stabilizing agents suppress cancer cell growth by promoting accelerated assembly of excessively stable microtubules, which consequently leads to cell cycle arrest in G₂-M and eventual cell death (1–5). Although the exact mechanism of apoptosis-induction is not fully understood (4, 6–8), these agents are of high interest due to their capacity to induce apoptosis even in p53-mutated, otherwise chemo- and radioresistant cells (9). For a combined treatment modality with ionizing radiation (IR), the search for chemotherapeutic agents specifically arresting the cell cycle at G₂-M is tempting because G₂-M is the most radiosensitive cell cycle phase (10–13). Therefore, the combined use of microtubule-stabilizing agents such as taxanes in combination with IR might be clinically interesting to improve local tumor control (14–16).

Despite its clinical success for some tumor entities, treatment with taxanes (e.g., paclitaxel) is often limited by taxane-related toxicities, such as neutropenia, peripheral neuropathy, and alopecia (17–19), as well as development of multidrug resistance which is often associated with overexpression of P-glycoprotein. This has led to the search for novel microtubule-stabilizing agents lacking these limitations of taxanes (3, 20).

Epothilones, although structurally unrelated to the taxanes, exert strong microtubule-stabilizing effects (2). Patupilone (epothilone B, EPO906) and epothilone A are naturally occurring secondary metabolites that are produced by myxobacteria and were first described for their selective antifungal activity (21). These tubulin-polymerizing and microtubule-stabilizing agents are competitive inhibitors of paclitaxel-binding to tubulin, sharing with the taxanes a partially overlapping binding site (1, 22–25). Patupilone is a more potent microtubule stabilizer and antiproliferative agent than either demethylated epothilone B (epothilone A), the lactam analogue of epothilone B (aza-epothilone B, also known as BMS-247550), or paclitaxel (2, 26, 27). More importantly, patupilone is least sensitive to P-glycoprotein efflux pump-mediated multidrug resistance (1, 26–28).

Based on the close functional similarity to taxanes and the cellular response leading to an accumulation of the cell population in the radiosensitive G₂-M phase of the cell cycle, epothilones are presumed to sensitize tumor cells to ionizing radiation (2, 10–13). Here we profile the combined effect of

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Requests for reprints: Martin Prusky, Laboratory for Molecular Radiobiology, Department of Radiation Oncology, University Hospital Zurich, Ramistr. 100, CH-8091 Zurich, Switzerland. Phone: 4112558549; Fax: 4112554435; E-mail: martin.prusky@usz.ch.

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the potent epothilone derivative patupilone with ionizing radiation against p53-deficient, paclitaxel-sensitive E1A/ras-transformed mouse embryo fibroblasts (MEF) and multi-drug-resistant p53-mutant human colon adenocarcinoma tumor cells at low concentration both *in vitro* and *in vivo*. As the role of G₂-M cell cycle arrest in the radiosensitization mechanism, especially at low concentrations of these agents, is still controversial, we also closely analyzed cell cycle progression at low concentrations of patupilone and IR alone and in combination. We show here that patupilone can sensitize both paclitaxel-sensitive as well as paclitaxel-resistant cells to ionizing radiation, and propose an S-phase progression-related mechanism for radiosensitization.

MATERIALS AND METHODS

Paclitaxel and verapamil hydrochloride were purchased from Calbiochem (San Diego, CA). Patupilone (epothilone B, EPO906) was provided by Novartis Pharma (Basel, Switzerland).

Cell Cultures and Irradiation. p53-/- MEFs were derived from 13.5-day-old embryos and stably transfected with the two oncogenes *E1A* and *T24 H-ras* as described in ref. 29. These transformed MEFs were used at low passage numbers and cultured at 5% CO₂ atmosphere in DMEM containing 10% FCS and 10% bovine calf serum supplemented with penicillin and streptomycin. SW480 human colon adenocarcinoma cells were cultured in RPMI 1640 supplemented with 10% FCS, penicillin, and streptomycin. Irradiation of cell cultures was carried out at room temperature using a Pantak Therapax 300-kV X-ray unit at 0.7 Gy/min.

Cell Proliferation and Clonogenic Survival Assay. Tumor cell proliferation was assessed in 96-well plates with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-like colorimetric Alamar Blue assay, which is based on detection of metabolic activity according to the protocol of the manufacturer (Biosource International, Camarillo, CA). Absorption was measured at 570 and 630 nm using a Tecan GENios spectrophotometer. To determine clonogenic survival, the number of single seeded cells was adjusted to obtain ~100 colonies per cell culture dish with a given treatment. After treatment with different regimens, cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and allowed to grow for 6 (*E1A/ras*-transformed MEFs) or 12 (SW480) days, respectively, before fixation in methanol/acetic acid (75%:25%) and staining with crystal violet. Only colonies with more than 50 cells/colony were counted. All proliferation and clonogenic assays were repeated as independent experiments at least twice. For combined treatment, cells were preincubated with patupilone, paclitaxel, or control solution 24 hours before irradiation. For *in vitro* experiments, patupilone and paclitaxel were dissolved in DMSO (1 mmol/L stock solution) and further diluted with media in the presence of 10% FCS. Verapamil, an inhibitor of the drug efflux protein P-glycoprotein, was dissolved in H₂O (1 mg/mL stock solution) and further diluted with FCS-containing media. For inhibition of this multidrug resistance-related efflux protein, cells were preincubated with verapamil 30 minutes before treatment with patupilone or paclitaxel.

Subcellular Fractionation and Immunoblotting. To obtain cytosolic and membranous cell constituents, subcellular fractionation of cell lysates was done essentially as described in ref. 30. Briefly, SW480 cells and transformed MEFs were cultivated in 10-cm dishes, washed twice with ice cold PBS, and then harvested by scraping the cells with 500 µL of precooled hypotonic lysis buffer (HEPES, pH 7.4, 25 mmol/L; β-glycerophosphate, 25 mmol/L; EDTA, pH 7.4, 2 mmol/L; leupeptin, 10 µg/mL; aprotinin, 10 µg/mL; Na₃VO₄, 1 mmol/L; phenylmethylsulfonyl fluoride, 1 mmol/L). The cell suspension was sonicated with 10 cycles (duty cycle, 50%; power output, 6) on ice using a tip sonicator and the cell lysate was centrifuged at 4°C for 15 minutes at 14'000 rpm. The supernatant (cytosolic fraction) was separated and the pellet was carefully washed with 1 mL of hypotonic lysis buffer and resuspended in 250 µL Triton lysis buffer (HEPES, pH 7.4, 25 mmol/L; β-glycerophosphate, 25 mmol/L; EDTA, pH 7.4, 2 mmol/L; leupeptin, 10 µg/mL; aprotinin, 10 µg/mL; Na₃VO₄, 1 mmol/L; phenylmethylsulfonyl fluoride, 1 mmol/L; Triton X-100, 1%). After a second sonication step, the sonicated lysate was centrifuged at 4°C for 15 minutes at 14'000 rpm, and the supernatant (clarified membrane fraction) was immediately frozen and stored at -70°C. The protein concentration was determined with the BioRad DC Protein assay (Bio-Rad Laboratories, Rheinach BL, Switzerland). Samples were separated by SDS-PAGE followed by Western blotting onto polyvinylidene difluoride membranes. Membranes were probed with the P-glycoprotein antibody anti-mdr (Ab-1, Calbiochem). Antibody detection was achieved by enhanced chemoluminescence (Amersham, Piscataway, NJ) according to the protocol of the manufacturer. All experiments were carried out independently at least thrice.

Cell Cycle Analysis. Cell cycle distribution was measured at distinct time points before (control) and after different treatment modalities. Bivariate distributions of 5-bromo-2'-deoxyuridine content (Sigma, Buchs SG, Switzerland) versus DNA content (propidium iodide) were analyzed according to the protocol of the manufacturers on the FITC-conjugated anti-5-bromo-2'-deoxyuridine antibody (Becton Dickinson Immuno-cytometry Systems, San Jose, CA). Analysis was done on an EPICS ELITE apparatus (Beckmann-Coulter). The data were analyzed using the MultiCycle (Phoenix Flow Systems, Inc., San Diego, CA) and WinMDI 2.8 software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA). At least two independent experiments in duplicate were done for each set of data. Statistical analysis was done with the Fisher's *t* test.

Tumor Xenograft in Nude Mice and Administration of Patupilone and Irradiations. Human colon carcinoma cells (SW480) were injected subcutaneously (4×10^6 cells) on the back of 4- to 8-week-old athymic nude mice. Tumor volumes were determined from caliper measurements of tumor length (*L*) and width (*l*) according to the formula $(L \times l^2) / 2$. Tumors were allowed to expand to a volume of $200 \text{ mm}^3 \pm 10\%$ before start of treatment. Using a customized shielding device, mice were given a strictly locoregional radiotherapy of $4 \times 3 \text{ Gy}$ at 4 consecutive days using a Pantak Therapax 300-kV X-ray unit at 0.7 Gy/min. Patupilone (dissolved in 30% polyethylene glycol-300:70% saline) was applied intravenously (at day 0 of the treatment) 24 hours before the first treatment with IR. Statistical Analysis of the *in vivo* data was done with the Mann-Whitney *U* test.

RESULTS

Antiproliferative Effect at Low Nanomolar Concentrations of Patupilone. The antiproliferative effect of patupilone was tested over 72 hours in genetically defined p53-deficient *E1A/ras*-transformed MEFs (Fig. 1A) and in the p53-mutated human colon adenocarcinoma cell line SW480 (Fig. 1B). Both cell lines were highly sensitive to patupilone; the SW480 tumor cells even responded in a subnanomolar range ($IC_{50} \sim 0.2$ nmol/L). A similar antiproliferative effect was observed in genetically identical but p53-wild-type *E1A/ras*-transformed MEFs (data not shown). Micromolar concentrations of epothilones are required for microtubule polymerization *in vitro*. However, epothilones also extensively accumulate inside cells and thus nanomolar concentrations of patupilone in the cellular media effectively translate into intracellular concentration levels sufficient for microtubule stabilization (2).

In parallel, the two cell lines were treated with increasing doses of paclitaxel. Whereas the *E1A/ras*-transformed cell line was sensitive to paclitaxel in the low nanomolar range (25–50 nmol/L, Fig. 1C), the SW480 tumor cells were resistant to paclitaxel up to 200 nmol/L (Fig. 1D), indicative for a paclitaxel-

specific, but not epothilone-specific, treatment resistance for microtubule inhibitors in this cell line.

Antiproliferative and Clonogenic Cell Death-Inducing Effect by Combined Treatment with Patupilone and IR. Treatment of cells with microtubule inhibitors can induce a cell cycle arrest in the G₂-M phase (2). Based on the concept that microtubule inhibitors induce an accumulation of cells in this most radiosensitive phase of the cell cycle, the antiproliferative effect of patupilone in combination with ionizing radiation was analyzed in tumor cells, which were continuously pretreated for 24 hours with patupilone before irradiation.

Proliferation assays with increasing concentrations of patupilone and IR were done in both p53-/- *E1A/ras*-transformed MEFs (Fig. 2A) and SW480 cells (Fig. 2B). An at least additive antiproliferative effect was induced with doses that show less than 50% inhibition of proliferation over 72 hours when used alone. Likewise, treatment with IR in combination with paclitaxel induced an additive effect in both cell lines, but again high nanomolar concentrations of paclitaxel were required to achieve this effect in the human SW480 tumor cell line (Fig. 2C and D).

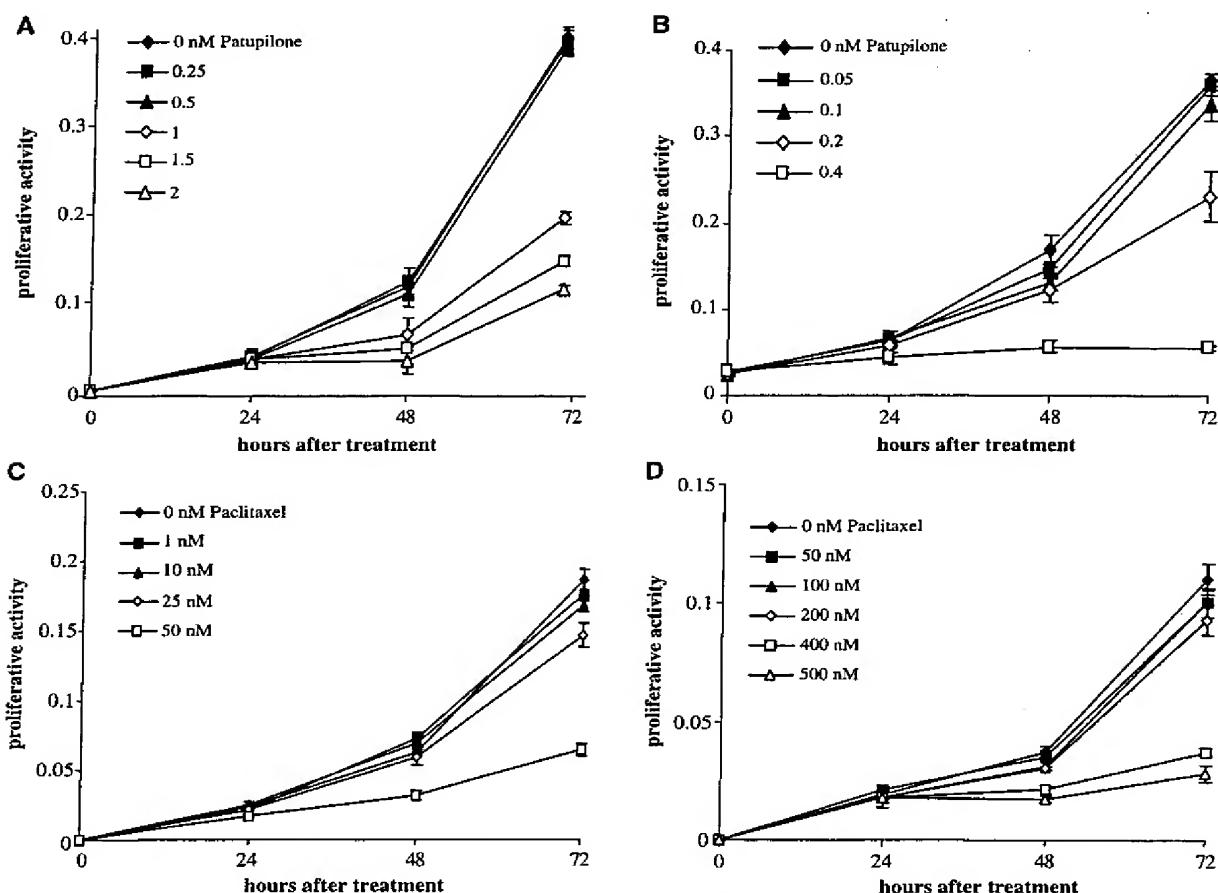


Fig. 1 Antiproliferative activity of patupilone and paclitaxel. p53-/- *E1A/ras*-transformed MEFs (A, C) and human colorectal p53-mutant SW480 cells (B, D) were treated with increasing doses of patupilone (A, B) or paclitaxel (C, D). The proliferative activity was determined at the indicated time points using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-like Alamar Blue assay.

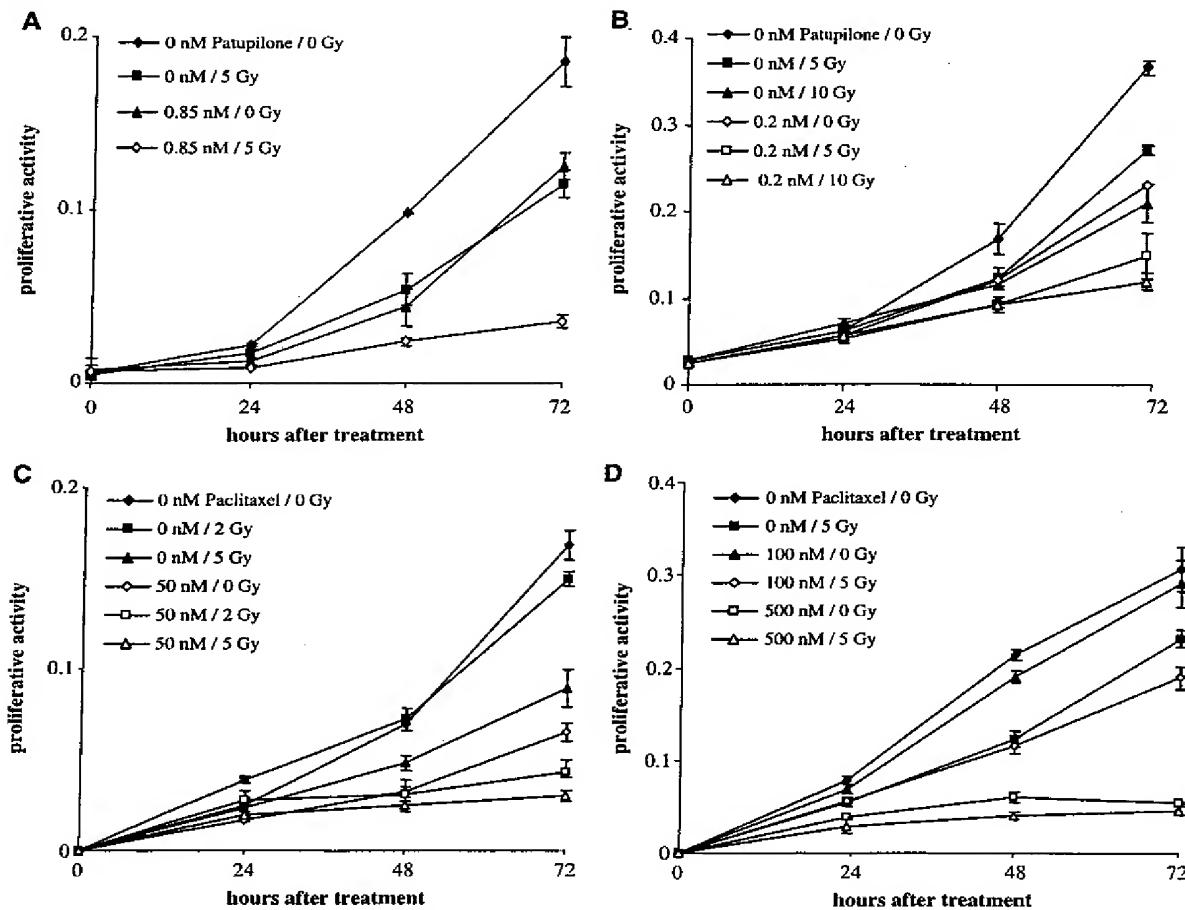


Fig. 2. Antiproliferative activity of patupilone or paclitaxel in combination with IR. p53-/- EIA/ras-transformed MEFs (*A*, *C*) and human colorectal p53-mutant SW480 cells (*B*, *D*) were treated with increasing doses of patupilone (*A*, *B*) or paclitaxel (*C*, *D*) in combination with IR. Both patupilone and paclitaxel were added 24 hours before irradiation. The proliferative activity was determined at the indicated time points using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-like Alamar Blue assay.

Next, clonogenic survival assays were done with increasing concentrations of patupilone alone and in combination with irradiation (2 and 5 Gy) and the cellular response to IR and patupilone was compared with the radiosensitizing effect of paclitaxel. Clonogenic survival of MEFs and SW480 tumor cells was reduced on treatment with increasing concentrations of patupilone alone, but SW480 cells were more sensitive to patupilone than the p53-deficient transformed MEFs at all concentrations tested (Fig. 3*A* and *C*). Paclitaxel reduced clonogenic survival more drastically in the EIA/ras-transformed MEFs than in the SW480 cells and again in a dose range of 50 to 500 times higher than patupilone, as already observed in the proliferation assay (Fig. 3*B* and *D*). Preincubation of cells with patupilone at dose levels with only a minimal antiproliferative effect sensitized both tumor cell populations to IR and clonogenic survival was supra-additively reduced (Fig. 3*A* and *C*). A supra-additive effect of paclitaxel and IR was also observed against the p53-deficient transformed MEFs with increasing concentrations of paclitaxel (25–50 nmol/L, Fig. 3*B*). However, almost no additivity was observed on SW480 cells

when IR was combined with paclitaxel at doses as high as 100 nmol/L paclitaxel (Fig. 3*D*). Overall these results show that whereas the oncogene-transformed MEFs are sensitive to both patupilone and paclitaxel, SW480 is a paclitaxel-refractory but patupilone-sensitive tumor cell line. Furthermore, patupilone has a supra-additive antiproliferative and clonogenic cell death-inducing effect when used in combination with IR.

The Multidrug Resistance-Related Efflux Pump P-Glycoprotein Is Overexpressed in the SW480 Cell Line and Renders SW480 Cells Paclitaxel-Resistant. Paclitaxel resistance in tumor cells is often due to overexpression of the multidrug resistance-related P-glycoprotein (as reviewed in ref. 31). Proliferation experiments were therefore done with SW480 cells, which were pretreated with the multidrug resistance modulator verapamil 30 minutes before paclitaxel treatment. Verapamil (5 µg/mL) resensitized SW480 cells to low doses of paclitaxel (10 nmol/L), indicative that enhanced P-glycoprotein activity is responsible for the paclitaxel-refractory effect in this cell line (Fig. 4*A*, compare with Fig. 1*D*). Verapamil alone did not have an antiproliferative effect at the applied concentration

and the antiproliferative effect of patupilone was mainly independent of verapamil. Western blotting indeed confirmed that P-glycoprotein was overexpressed in SW480 tumor cells in comparison with the paclitaxel-sensitive *E1A/ras*-transformed MEFs (Fig. 4B). The verapamil-dependent antiproliferative effect of paclitaxel in the SW480 cells was also tested in combination with increasing doses of IR (Fig. 4C). A strong supra-additive inhibitory effect on proliferative activity was determined on combined treatment with paclitaxel (10 nmol/L) and IR (2 and 5 Gy, respectively) but only in cells which were pretreated with the multidrug resistance modulator verapamil.

The Radiosensitizing Effect of Patupilone Is Not Mediated by G₂-M Phase Accumulation. Both microtubule inhibitors and IR can modulate cell cycle progression. The cooperative effect of their combined treatment has been linked to the G₂-M cell cycle arrest induced by microtubule inhibitors. We therefore analyzed cell cycle alterations at multiple time points in response to the different treatment modalities and especially with regard to the low concentration of patupilone (0.25 nmol/L) used for the combined treatment regimen. Pretreatment of SW480 cells with patupilone at this low concentration did not alter the G₂-M cell cycle distribution over 34 hours (Fig. 5A) or 38 hours (Fig. 5B), as determined by bivariate distribution analysis of DNA synthesis (5-bromo-2'-deoxyuridine incorporation) versus DNA content (propidium iodide amount). Extended accumulation of cells in the G₂-M phase was only observed after treatment with patupilone above 10 nmol/L

(data not shown). On the other hand, IR alone induced a significant G₂-M arrest at the 10-hour time point ($P = 0.001$, control versus IR) and was again decreasing at the 14-hour time point after irradiation. Surprisingly, combined treatment of patupilone and IR induced a lower accumulation of cells in G₂-M at the 10-hour time point in comparison with the IR-induced G₂-M arrest (Fig. 5A, $P = 0.001$, IR versus combined) which was not further increased at the 14-hour time point and still below the IR-induced G₂-M arrest (Fig. 5B).

A loss of cells in G₁ paralleled these G₂-M-related cell cycle alterations (not shown). Close examination of the S-phase cell cycle distribution indicated a tendency that patupilone treatment induces a transient accumulation of cells in this phase (Fig. 5C and D), which was significantly enhanced in response to combined treatment with IR (Fig. 5C, $P = 0.002$, control versus combined; D, $P = 0.003$) but was minor in response to IR alone (Fig. 5C, $P = 0.008$, IR versus combined; D, $P = 0.005$). Thus, the combined treatment effect of patupilone and IR as reported here is not due to a microtubule inhibitor-induced accumulation of cells in the radiosensitive G₂-M phase but rather due to an S-phase-related process. Analysis of a subG₁-population did not reveal any significant changes, suggesting that apoptosis is not involved in these p53-mutated, radiation-resistant adenocarcinoma cells in response to these treatment modalities (data not shown).

Radiosensitizing Effect of Patupilone on Tumor Xenografts. Based on the at least additive effects of combined

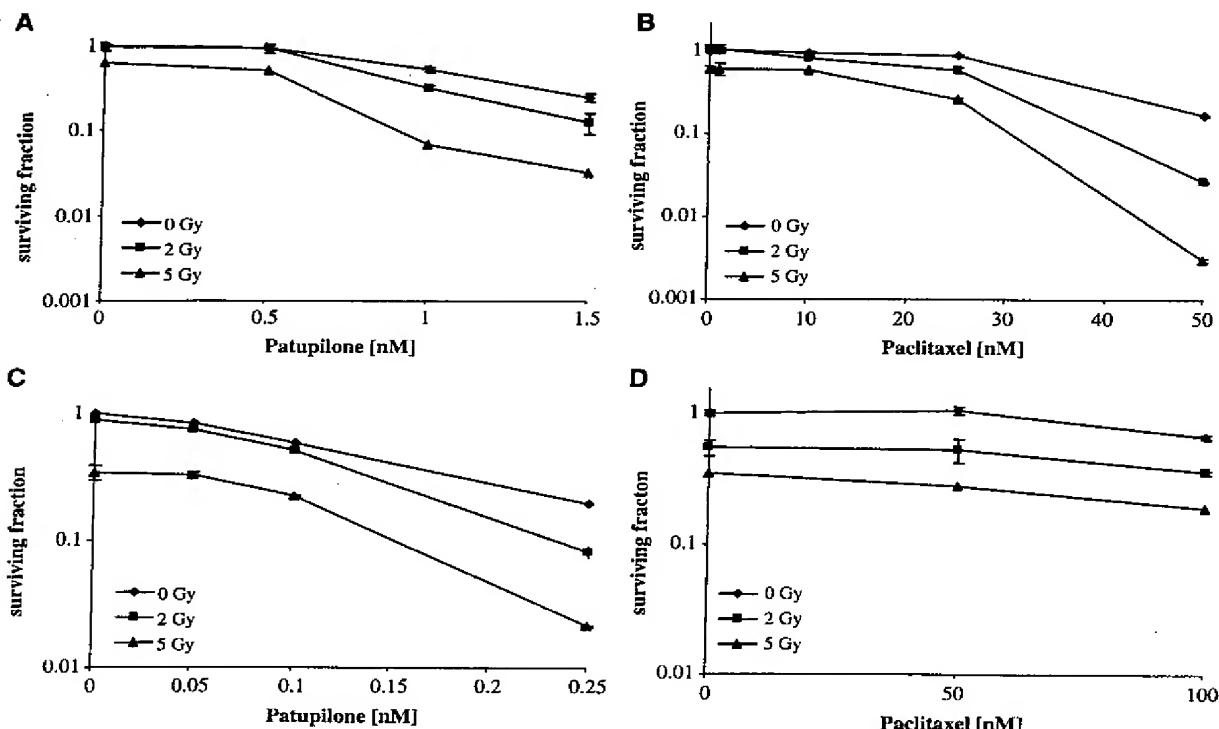


Fig. 3 Clonogenic survival of cells after treatment with either patupilone or paclitaxel in combination with IR. p53-/- *E1A/ras*-transformed MEFs (A, B) and SW480 cells (C, D) were treated with patupilone (A, C) or paclitaxel (B, D) alone and in combination with IR. Results of a representative experiment ($n = 2$).

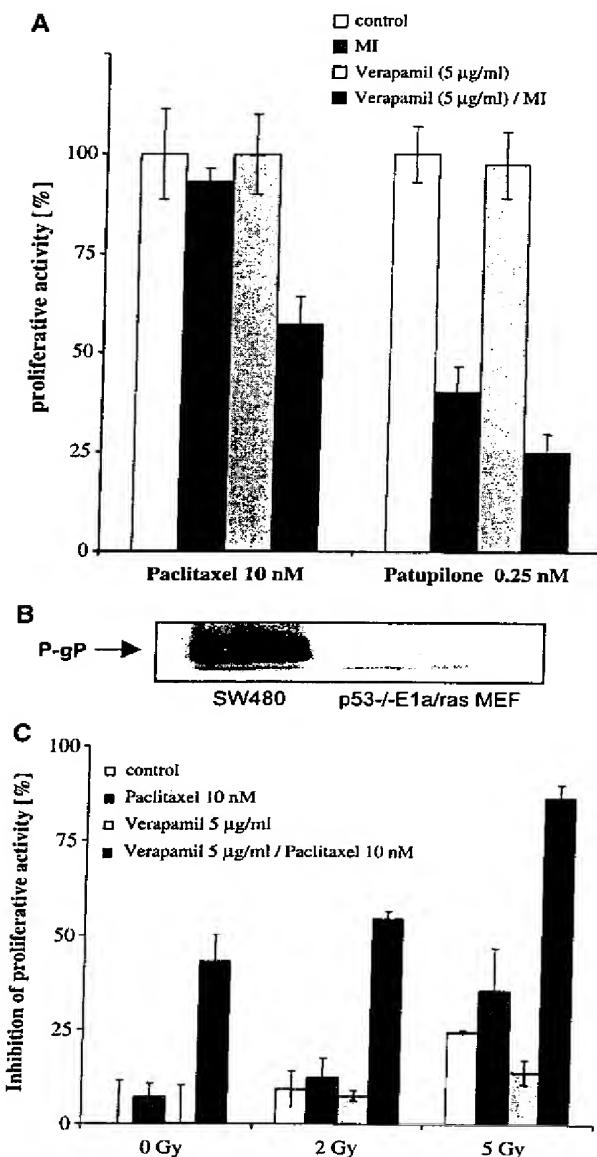


Fig. 4 Antiproliferative activity of paclitaxel after treatment with verapamil. **A**, SW480 cells were preincubated with verapamil (5 μg/ml) for 30 minutes and proliferative activity was determined 24 hours after treatment with the microtubule inhibitors (MI) paclitaxel or patupilone. **B**, P-glycoprotein expression was determined by Western blotting in the subcellular membrane fraction of SW480 cells and E1a/ras-transformed MEFs. **C**, SW480 cells were pretreated with verapamil as in **A** and irradiated 24 hours after paclitaxel treatment. Proliferative activity was determined 24 hours after treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-like Alamar Blue assay. The proliferative activity of untreated cells was set as 100%; inhibition of proliferative activity in untreated cells was set as 0%.

treatment *in vitro*, a combined treatment regimen with patupilone and IR was tested *in vivo* against paclitaxel-resistant tumors derived from human adenocarcinoma SW480 cells, s.c. injected into the back of nude mice. The solvent (polyethylene

glycol-300/saline) for i.v. administration of patupilone, which is less toxic than the chremophore usually used for preclinical *in vivo* experiments with paclitaxel, was included as vehicle in control and IR-treated groups. Treatment was started when tumors reached a minimal size of $200 \text{ mm}^3 \pm 10\%$ (day 12–17 after cell injection).

In vivo studies were done with locoregional application of IR using a shielding device and fractionated single doses of 3 Gy. This daily dose is applied when fractionated radiotherapy is used for the treatment of human malignancies. For practical reasons, only four fractions were chosen as treatment regimen, but the response to such a regimen has been previously found to be useful for treatment evaluation (32, 33). Figure 6 summarizes the effect of tumor treatment with patupilone alone (2 mg/kg once), IR alone (vehicle combined with $4 \times 3 \text{ Gy}$), and in combination (2 mg/kg once combined with $4 \times 3 \text{ Gy}$), in comparison with a vehicle alone–treated control group. Patupilone was applied 24 hours before the first of four fractions of irradiation applied on 4 consecutive days. Determination of treatment-related body weight changes only revealed a minor patupilone-dependent transient weight loss over 48 hours after patupilone application (<10%, data not shown) and no skin changes or tissue damage were observed in the co-irradiated healthy tissue area around the tumor during the follow-up period of tumor growth. Treatment with patupilone or IR alone resulted in a partial tumor growth suppression whereas combined treatment exerted a strong tumor growth control during treatment and the follow-up period ($P = 0.0004$, IR versus combined treatment). Overall these results show that patupilone might be a promising alternative in multidrug-resistant tumors for a combined treatment regimen using microtubule inhibitors and IR.

DISCUSSION

The search for novel microtubule inhibitors devoid of taxane-associated liabilities revealed multiple structurally distinct microtubule-stabilizing compounds, which are currently tested at the preclinical and clinical level as single agents or as part of a combined anticancer treatment modality (reviewed in ref. 34). Here we have examined the effect of the novel microtubule inhibitor patupilone alone and in combination with IR and determined at least additive antiproliferative and cytotoxic responses against p53-defective, radiation-resistant tumor cell lines. Importantly, patupilone at subnanomolar concentrations alone and in combination with IR retained a strong antitumor effect against paclitaxel-resistant, P-glycoprotein-overexpressing human SW480 adenocarcinoma cells. Whereas treatment of SW480-derived xenografts in nude mice with patupilone or IR alone resulted in a partial tumor growth delay, a low-dose combined treatment regimen exerted a strong and significant tumor growth arrest.

Patupilone is currently tested in clinical phase I/II trials and already shows a broad antitumor activity. More importantly, patupilone is also able to induce responses in taxane-resistant cases (reviewed in ref. 34). Our own preclinical studies now show that patupilone is also able to sensitize taxane-resistant tumors to IR, and thus such a combined treatment modality qualifies for clinical trials in P-glycoprotein (*MDR1*)-overexpressing tumors. For example, our experiments were done with the human colon

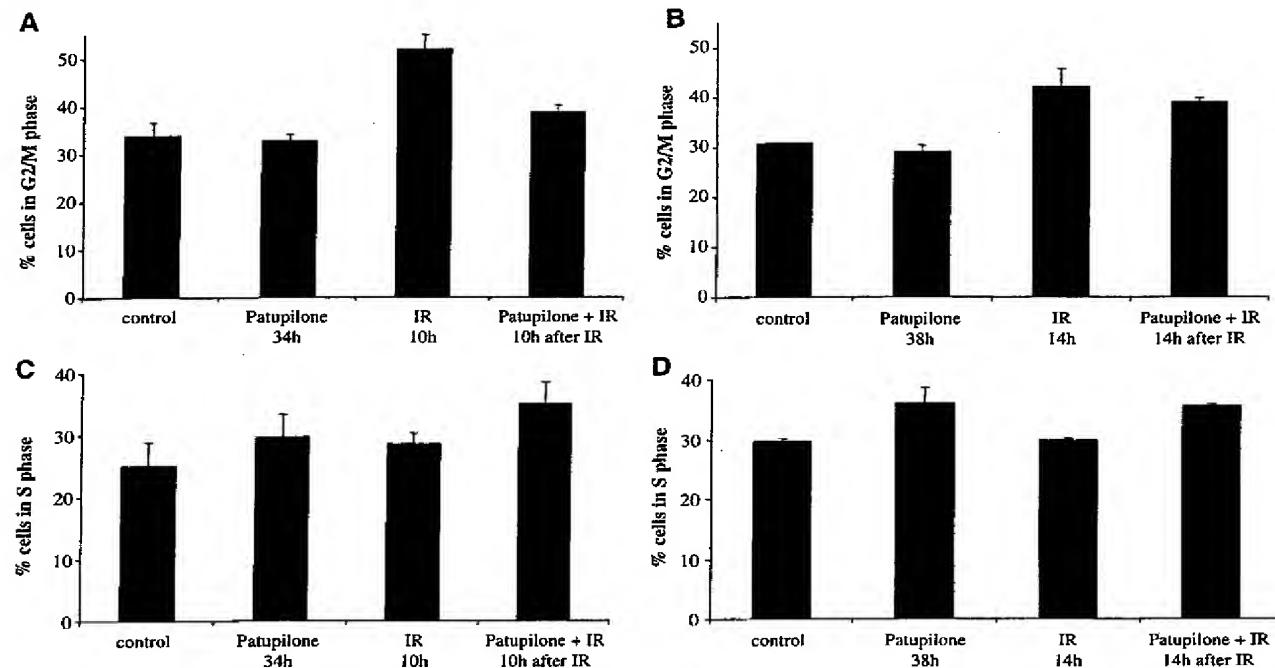


Fig. 5 Cell cycle distribution of SW480 cells after treatment with patupilone or IR, alone or in combination. SW480 cells were treated with patupilone (0.25 nmol/L), IR (5 Gy), alone or in combination, and cell cycle distribution was determined by bivariate flow cytometry. Columns, percentage of cells distributed in G₂-M phase (*A*, *B*) and S phase (*C*, *D*). Cells were preincubated with patupilone for 24 hours before irradiation and analyzed at the indicated time points. Data are representative of at least two independent experiments.

adenocarcinoma cell line SW480. A strong link exists between *MDR1* overexpression and tumorigenesis of colorectal cancer. Further, *MDR1* plays an important role in tumor progression in at least one proportion of colorectal cancer with high microsatellite instability (35, 36).

Microtubule inhibitors modulate microtubule stability and block the degradation and breakdown of the mitotic spindle apparatus which eventually leads to cell cycle arrest

before completion of mitosis. Patupilone and paclitaxel induce this G₂-M arrest at high concentrations of 100 nmol/L and 1 μmol/L, respectively (2). Interestingly, in our experiments, we observed a radiosensitizing, supra-additive effect of patupilone with IR at concentrations of patupilone that did not lead to accumulation of cells into the radiosensitive G₂-M phase. As expected, treatment with IR alone resulted in an extended accumulation of SW480 cells in the G₂-M phase (37, 38). Detailed cell cycle analysis revealed that this effect was markedly reduced when cells were pretreated with a radiosensitizing concentration of 0.25 nmol/L patupilone. On the other hand, an enhanced accumulation of cells in S phase on combined treatment suggests that a decelerated S-phase transgression-related mechanism might be responsible for the supra-additive effect of patupilone in combination with IR. Thus, the radiosensitizing mechanism of microtubule inhibitors is not necessarily only related to a microtubule inhibitor-mediated G₂-M block as previously stated for paclitaxel (16). Interestingly, low concentrations of microtubule-stabilizing agents that are not associated with a G₂-M cell cycle block have been previously noted to cause cellular effects that may in part explain the radiosensitization reported here (e.g., low concentrations of epothilone B lead to aberrant mitosis and aneuploid cells in a manner that was associated with increased expression of stress genes and CD95; ref. 39).

Multiple classes of agents exist that enhance IR-induced cell killing in S phase by different mechanisms. For example, 5-bromo-2'-deoxyuridine increases the amount of DNA damage and at the same time decreases the rate of DNA repair

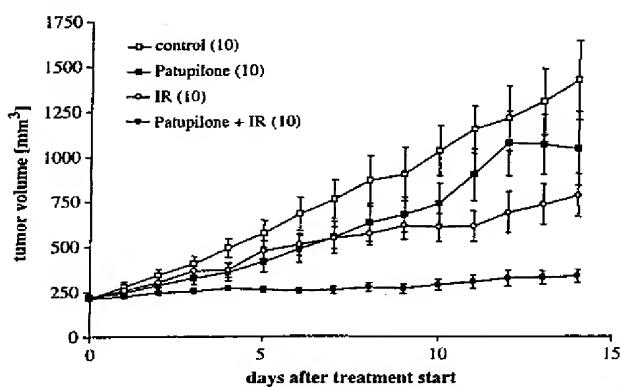


Fig. 6 The effect of patupilone and IR alone or in combination on the growth of SW480-derived xenografts in nude mice. Mice were treated with patupilone (2 mg/kg once) and IR (4 × 3 Gy, on 4 consecutive days), alone and in combination, with administration of patupilone or the vehicle 24 hours before the first fraction of IR. Points, mean ($n = 10$); bars, SE.

(40). The clinically relevant nucleoside analogue gemcitabine (*2',2'-difluoro-2'-deoxycytidine*) neither increases double-strand breaks nor decreases the rate of their repair (41, 42) but gemcitabine-mediated decrease in the dATP pool is relevant for S-phase-dependent radiosensitization (43, 44). Further genetic elements which activate an S-phase checkpoint, like enhanced expression of the transcription factor E2F-1, also enhance the cytotoxic effect of IR (45). Research on microtubules has mainly focused on its role during G₂-M and has strongly neglected other phases of the cell cycle thus far. Interestingly though, microtubule-associated proteins have been discovered which play a role in the cellular stress response to IR also in other stages of the cell cycle. For example, the microtubule-associated protein GTSE-1 is specifically expressed during S and G₂ phases of the cell cycle in response to DNA damage and controls stress-induced apoptosis (46). Eventually disturbance of microtubular integrity by microtubule inhibitors might affect the function of this DNA-damage response element or other microtubule-associated proteins. Own future studies will investigate the mechanism of low-dose patupilone radiosensitization during S phase and will in particular focus on the interference with the DNA repair machinery (47, 48).

To our knowledge, this is the first report investigating patupilone in combination with IR *in vitro* and *in vivo*. A previous report showed that the semisynthetic aza-epothilone derivative BMS-247550 is as potent as paclitaxel against human lung cancer cells *in vitro* and synergistically interacts with IR (49). The strong shift into the radiosensitive G₂-M cell cycle phase induced by BMS-247550 alone was proposed to be the mechanism for radiosensitization in the apoptosis-sensitive cell line evaluated. As outlined in this report, patupilone is much more potent than the taxane in both apoptosis- and radiation-resistant cell lines and reduces clonogenic survival in combination with IR independent of a G₂-M arrest *in vitro*. Although difficult to compare, combined treatment of IR with a single administration of patupilone *in vivo* seemed to result in a stronger tumor growth suppression than that reported for BMS-247550 (49).

Precclinical *in vivo* studies using patupilone as monotherapy (1 × 4 and 3 × 4 mg/kg) resulted in growth inhibition or tumor regression in several tumor models of lung, breast, colon, and prostate tumor xenografts including P-glycoprotein-overexpressing, paclitaxel-resistant KB-8511 tumor xenograft model (2). As determined in this report, combined treatment with a single, well-tolerated dose of patupilone followed by four fractionated administrations of IR exerted surprisingly strong tumor growth suppression in a colon adenocarcinoma model. Interestingly, patupilone is antiproliferative and induces apoptosis in microvascular and macrovascular endothelial cells also when applied in a low-dose treatment schedule (50). Especially in case of a single administration of patupilone the "metronomic" low-dose concentration remaining in the tumor circulation over time might still target the tumor vasculature. Thus, in parallel to its antitumor cell-directed effect, patupilone is also be considered antiangiogenic. Combined treatment of IR with inhibitors of angiogenesis is a promising strategy and has been tested with multiple antiangiogenic compounds (32, 51, 52), mainly directed against the vascular endothelial growth factor receptor-controlled endothelial cell signaling. Thus, IR

as well as patupilone can target both the tumor tissue and the tumor vasculature and thus may cooperate on different tissue levels, which in turn may further enhance the overall antitumor effect of this combined modality.

Overall our *in vitro* and *in vivo* results suggest a multilayered synergistic response on the cellular and tumor tissue levels induced by IR and patupilone. The strong treatment response suggests the combination of patupilone and IR as a promising combined treatment modality especially in the treatment of multidrug-resistant paclitaxel-refractory tumor entities.

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Role of the Microenvironment for Radiosensitization by Patupilone

Carla Rohrer Bley,^{1,3} Wolfram Jochum,² Katrin Orlowski,¹ Polina Furmanova,¹ Van Vuong,¹ Paul M.J. McSheehy,⁴ and Martin Prusky¹

Abstract **Purpose:** The combined treatment modality of ionizing radiation (IR) and the clinically relevant microtubule-stabilizing compound patupilone (epothilone B, EPO906) is a promising approach for anticancer therapy. Here, we investigated the role of the tumor microenvironment for the supra-additive *in vivo* response in tumor xenografts derived from patupilone-sensitive and patupilone-resistant non-small cell lung cancer cells.

Experimental Design: The treatment response to a combined regimen of patupilone and IR was investigated *in vitro* and in tumor xenografts derived from wild-type A549 and A549.EpoB40 cells, which are resistant to patupilone due to a β -tubulin mutation.

Results: In both A549 and A549.EpoB40 cells, proliferative activity and clonogenicity were reduced in response to IR, whereas patupilone, as expected, inhibited proliferation of the mutant cell line with reduced potency. Combined treatment with patupilone and IR induced a cytotoxic effect *in vitro* in an additive way in A549 cells but not in the tubulin-mutated, patupilone-resistant A549.EpoB40 cells. A supra-additive tumor growth delay was induced by combined treatment in xenografts derived from A549 cells but not in xenografts derived from A549.EpoB40 cells. Histologic analysis revealed a significant decrease in tumor cell proliferation (Ki-67) and microvessel density and a treatment-dependent change of tumor hypoxia in A549 but not A549.EpoB40 xenografts.

Conclusions: Using a genetically defined patupilone-sensitive and patupilone-resistant tumor model, we here showed that the major cytotoxic effect of the combined treatment modality of IR and patupilone is directed against the tumor cell compartment. The induced antiangiogenic effect derives indirectly from the tumor cell.

Interference with microtubule function is a promising strategy for anticancer therapy (1). This approach has been extensively validated by the use of taxanes (microtubule stabilizers) for the treatment of a wide variety of human malignancies. The suppression of microtubule dynamics interferes with mitotic spindle formation, leading to cell cycle arrest in M phase and eventually to apoptosis or postmitotic cell death. The primary mechanism of microtubule-stabilizing agents (MSA) at the biochemical level is well investigated, but the signaling consequences relevant for their cytotoxic effect are far from clear. Furthermore, treatment with taxanes (paclitaxel and docetaxel) is limited by taxane-related toxicities and the development of multidrug resistance. This has prompted an

ongoing worldwide search for novel microtubule-targeting compounds. One new class of MSAs are the epothilones, which are structurally distinct from the taxanes and may overcome some of their limitations, suggesting a promising new treatment approach for cancer (2–4).

Epothilones are nontaxoid MSA of bacterial origin, which share the same binding site on β -tubulin with taxanes (5, 6). One of the epothilones, patupilone (epothilone B, EPO906), is currently in phase II clinical development in several different solid tumors and is in phase III clinical trials for ovarian cancers in patients refractory to carboplatin-taxane treatment. Patupilone retains activity against P-glycoprotein-expressing multidrug-resistant cells both *in vitro* and *in vivo* (5) and shows different clinical toxicities to the taxanes (6, 7). Apart from the direct tumor-cytotoxic action, patupilone has also shown antivascular (8) and antiangiogenic (9) effects. The antiangiogenic effect might be linked to different mechanisms either directly targeting endothelial cells (10) or indirectly interfering with the secretion of proangiogenic agents from tumor cells (11, 12).

The accumulation of cells in the most radiosensitive G₂-M phase of the cell cycle represents the current rationale to combine MSAs with ionizing radiation (IR) and promises a strong radiosensitizing effect in combination with IR (2, 13, 14). Our own previous *in vitro* and *in vivo* experiments done with patupilone in combination with IR showed an at least additive cytotoxic effect in various cancer cell types *in vitro* and strong supra-additivity of the combined treatment regimen against tumor xenografts *in vivo*. Interestingly, also a S-phase

Authors' Affiliations: Departments of ¹Radiation Oncology and ²Pathology, University Hospital Zurich; ³Section of Radio-Oncology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland; and ⁴Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland

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Requests for reprints: Martin Prusky, Department of Radiation Oncology, University Hospital Zurich, Raemistr. 100, CH-8091 Zurich, Switzerland. Phone: 41-44-255-8549; Fax: 41-44-255-4435; E-mail: martin.prusky@usz.ch.

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Translational Relevance

Patupilone is a novel microtubule inhibitor with known antitumor and antiangiogenic/vascular activity currently in phase II/III clinical trials. Herein, we present first complementary *in vitro* and *in vivo* results investigating the role of the tumor microenvironment in the treatment response to the combination of ionizing radiation (IR) and patupilone. Using a genetically defined patupilone-sensitive and patupilone-resistant human non-small cell lung cancer tumor model system, we show that the major cytotoxic effect of the combined treatment modality of IR and patupilone is directed against the tumor cell compartment and that the induced antiangiogenic effect, which contributes to the synergistic treatment response of this combined treatment modality, derives indirectly from the tumor cell. These pre-clinical experiments are of relevance for clinical strategies that target the microtubule cytoskeleton and hypoxia-inducible factor dysregulation in combination with IR.

progression-related mechanism for radiosensitization was observed (15).

The observed *in vivo* supra-additive response to patupilone in combination with IR points to an additional mechanism of sensitization, which is not limited to the tumor cell but may also be due to effects on the microenvironment within the solid tumor (15).

Here, we investigated the different treatment modalities of patupilone and IR alone and in combination *in vitro* and *in vivo* using a patupilone-sensitive A549 cell line and the mutant derivative cell line (A549.EpoB40), which is strongly resistant to patupilone *in vitro* because it contains a defined β -tubulin mutation (3, 11, 16, 17). In this manner, we aimed to distinguish between a tumor cell and/or tumor microenvironment-directed effect of the different treatment modalities. Our results show a major tumor cell-directed effect with a subsequent indirect antiangiogenic effect especially by the combined treatment modality of patupilone in combination with IR.

Materials and Methods

Patupilone (epothilone B, EPO906) was provided by the Chemistry Department of Novartis Pharma. The human cell line pair A549 and A549.EpoB40 was obtained from the laboratory of Dr. Susan Band Horwitz.

Cell cultures, drug preparation, and irradiation. The human non-small cell lung cancer lines A549 and A549.EpoB40 were grown in RPMI 1640 containing 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, and 1% (v/v) L-glutamine at 37°C in 5% CO₂. A549.EpoB40 is derived from A549 by stepwise selection with patupilone and contains a point mutation in class 1 β -tubulin (β 292 from Gln to Glu), which is associated with ~95-fold resistance to patupilone (3). The A549.EpoB40 cell line was grown and maintained in medium containing 10 nmol/L patupilone. For *in vitro* experiments, patupilone was dissolved in DMSO (1 mmol/L stock solution) and further diluted with medium containing 10% FCS.

Irradiation of cell cultures was carried out using a Pantak Therapax 300 kV X-ray unit at 70 cGy/min at room temperature. For combined treatment, the cells were pretreated with patupilone 18 h before irradiation.

Cell proliferation and clonogenic survival assays. The proliferative activity of tumor cells was assessed in 96-well plates with the colorimetric alamarBlue assay, which is based on detection of metabolite activity according to the protocol of the manufacturer (Biosource International). Absorption was measured at 570 and 630 nm using a Tecan GENios spectrophotometer. Experiments were carried out under normoxic as well as hypoxic conditions. To render cells hypoxic, cells were grown in a hypoxic incubator at 1% pO₂. Clonogenic survival was determined by the ability of single cells to form colonies *in vitro*. The number of plated cells was adjusted to obtain ~100 colonies per cell culture dish with a given treatment. After treatment with different regimes, the dishes were maintained at 37°C in 5% CO₂ and allowed to grow for 12 days before fixation in methanol/acetic acid (3:1) and staining with crystal violet. Colonies with >50 cells/colony were counted manually. All assays were repeated as independent experiments at least thrice.

Tumor xenograft in nude mice and application of treatment regimes. A549 or A549.EpoB40 cells (4×10^6) were injected subcutaneously on the back of 4- to 8-week-old athymic nude mice. Tumor volumes were determined from caliper measurements of tumor length (*L*) and width (*W*) according to the formula: $(L \times W^2) / 2$. Tumors were allowed to expand to a volume of 200 mm³ ($\pm 10\%$) before treatment start. Using a customized shielding device, mice were given a strictly locoregional radiotherapy of 4 \times 3 or 3 \times 1 Gy at 4 or 3 consecutive days using a Pantak Therapax 300 kV X-ray unit at 0.7 Gy/min. Patupilone (dissolved in 30% PEG-300/70% saline) was applied intravenously 24 h before the first treatment with IR (at day 0 of the treatment). Tumor growth was monitored at least thrice and body weight once weekly.

Histology and immunohistochemistry. Tissues were immersion fixed in 4% PBS-buffered formalin and embedded in paraffin. Sections (3 μ m thick) were mounted on glass slides (SuperFrost Plus; Menzel), deparaffinized, rehydrated, and stained with H&E using standard histologic techniques. In addition, CC1 (antigen retrieval solution; Ventana Medical Systems)-pretreated sections were immunostained for Ki-67 (rabbit clone SP6; dilution 1:100; NeoMarkers), CD31 (rabbit polyclonal, ab28364; dilution 1:50; Abcam), and GLUT1 (rabbit polyclonal, MYM AB 1351; dilution 1:1,000; Chemicon International) using a Discovery immunohistochemistry staining system (Ventana Medical Systems). Detection of primary antibody was done with a biotinylated anti-rabbit IgG antibody (Jackson ImmunoResearch) and the iView DAB kit (Ventana Medical Systems). For hypoxia detection, pimonidazole hydrochloride (60-80 mg/kg) was applied intravenously 45 min before sacrifice. CC1-pretreated sections were incubated with Hypoxyprobe-1 MAb1 (mouse IgG1; dilution 1:300; Chemicon International) conjugated with FITC. Detection of primary antibody was done with a biotinylated mouse anti-FITC antibody (Jackson ImmunoResearch) and the iView DAB kit. All immunostains were counterstained with hematoxylin.

To determine proliferative activity, Ki-67-positive tumor cells were counted in 5 to 10 randomly chosen visual fields (magnification $\times 200$) in each xenograft ($n = 4$ for each group). The mean Ki-67-positive cell count from these fields was determined. Microvessel density (MVD) was determined in 5 to 10 randomly chosen visual fields (high-power fields) in each of three equally treated vital tumor tissues at $\times 100$ magnification (0.3 mm² visual field size). Hypoxia staining was quantified by manual counting using a grid of 1,200 points in two photographs per tumor, which displayed the whole tumor section.

Transfections and reporter gene assay. Both cell lines were stably transfected with a vector construct containing two copies of the Transferrin promoter hypoxia response element (HRE) and SV40 promoter in front of the firefly luciferase gene. Stably transfected, hygromycin-selected A549 and A549.EpoB40 cell lines were tested for luciferase enzymatic activity with a commercial kit (Bright-Glo luciferase assay; Promega) using a GloMax 20/20 luminometer. Hypoxic conditions were mimicked using dimethyloxaloylglycine (Biomol), a prolyl-4-hydroxylase inhibitor, which prevents hypoxia-inducible

factor (HIF) degradation at prior tested nontoxic concentrations of 0.25 and 1 nmol/L, respectively. To discriminate between treatment-induced cell death and treatment-interfered HIF signaling, cells were transiently cotransfected with a *Renilla* luciferase-expressing control vector. Patupilone was applied 24 h after transient transfection. Dimethyloxaloylglycine treatment was started 24 h and irradiation (5 Gy) 28 h after patupilone treatment. Luciferase activity was determined 14 h after treatment with dimethyloxaloylglycine.

Statistical analysis. Statistical analysis of the *in vivo* tumor growth data was done with the Mann-Whitney *U* test. The absolute tumor growth delay was defined as the time for tumor volume in the treated groups to triplicate the initial treatment size minus the time in the untreated control group to reach the same size. Kruskal-Wallis one-way ANOVA was used to test for significant differences of Ki-67, CD31, and hypoxia following the various treatments. Treatments effects (difference between control and treatments) were analyzed using the Mann-Whitney *U* test. The level of significance was set at 0.05. The calculations were all done using the StatView program (SAS Institute) version 5.0.1.

Results

Antiproliferative effect of patupilone under normoxic and hypoxic conditions. The antiproliferative effect of patupilone was tested over 72 h in the A549 wild-type and A549.EpoB40 mutant cell lines under normoxic and hypoxic conditions. A dose-dependent decrease of the proliferative activity was observed in the A549 tumor cells ($IC_{50} = 0.2$ nmol/L), whereas a >100-fold higher concentration (>20 nmol/L) was required to obtain the same antiproliferative effect in the mutant A549.EpoB40 cell line. No difference with regard to the antiproliferative effect by patupilone was observed under normoxia and under hypoxia (1% pO₂; Fig. 1A and B).

Antiproliferative and clonogenic cell death-inducing effect by combined treatment with patupilone and IR. The antiproliferative effect of patupilone in combination with IR was tested using increasing concentrations of patupilone and doses of IR. In the patupilone-sensitive A549 cell line, an additive antiproliferative effect was induced with 5 Gy, a dose that shows <50% inhibition of proliferation over 72 h in response to irradiation alone (Fig. 2A and B). No radiosensitizing effect could be detected in the drug-resistant A549.EpoB40 cell line at various doses of patupilone and IR. Interestingly, these cells were also slightly more radioresistant than the wild-type counterpart cell line (Fig. 2C and D). Patupilone was also highly potent against human umbilical vein endothelial cells. Picomolar concentrations of patupilone were sufficient to inhibit endothelial cell proliferation and cell viability alone and in combination with IR (data not shown). Clonogenic survival assays were done with both cell lines and increasing concentrations of patupilone and IR. Clonogenicity was reduced in an at least additive way in the A549 cell population. In contrast, the mutant cell line A549.EpoB40 was refractory to patupilone alone up to 10 nmol/L, and patupilone did not sensitize for IR (Fig. 2E and F).

Effect of combined treatment with patupilone and IR on growth of tumor xenografts. We previously observed a supra-additive effect of a combined patupilone/IR treatment on tumor growth delay in xenografts derived from radioresistant SW480 human colon adenocarcinoma cells (15). In the tumor xenografts derived from the wild-type A549 cell line, a small growth delay was induced by 3 × 1 Gy irradiation compared with untreated control tumors. Growth was significantly inhibited by patipi-

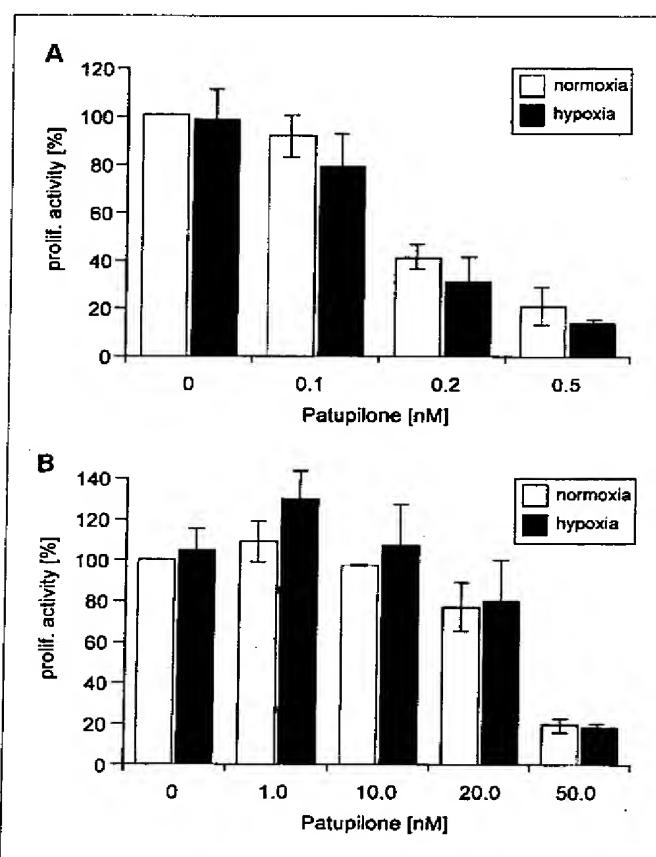


Fig. 1. Antiproliferative effect of patupilone under normoxic and hypoxic conditions. Lung adenocarcinoma cells A549 (A) and mutant cells A549.EpoB40 (B) were exposed to increasing concentrations of patupilone and grown in normoxic or hypoxic conditions (1%) for 72 h. Data were pooled from three independent experiments. Columns, mean; bars, SE.

lone (1 × 2 mg/kg) treatment alone ($P = 0.022$). In response to the combined treatment modality, a supra-additive tumor growth delay was observed. Tumor growth in the combined treatment group was significantly inhibited compared with IR- or patupilone-treated groups ($P = 0.010$ and 0.013; Fig. 3A). The absolute growth delay to triple the initial tumor volume at the start of treatment ($200 \text{ mm}^3 \pm 10\%$) was most enhanced with the combined treatment modality when compared with the absolute tumor growth delay in response to patupilone or IR alone [26 days (patupilone + IR) versus 6 days (patupilone) and 2 days (IR)], respectively (data not shown). In contrast, no tumor growth delay was induced by the same dose of patupilone in tumors derived from the drug-resistant A549.EpoB40 cells, indicating that the tumor growth suppression effect of patupilone primarily derives from tumor cell-directed cytotoxicity (Fig. 3B). The tumors derived from the mutated cell line were also more radiation-resistant than the wild-type counterparts. Nevertheless, a treatment regimen of 4 × 3 Gy alone induced a significant growth delay in tumors derived from these cells ($P = 0.004$). On the other hand, combined treatment did not further increase the antitumor effect, and even a slightly diminished absolute tumor growth delay was observed after combined treatment versus treatment

with IR alone [14 days (IR) versus 9 days (patupilone + IR); data not shown]. These results indicate that the major antitumor effect of the investigated treatment modality derives from tumor cell-directed cytotoxicity. A direct antiangiogenic effect of

patupilone alone or in combination with irradiation is unlikely to contribute to the observed tumor growth delay. Determination of body weight changes only revealed a minor patupilone-dependent transient weight loss over 48 h after treatment

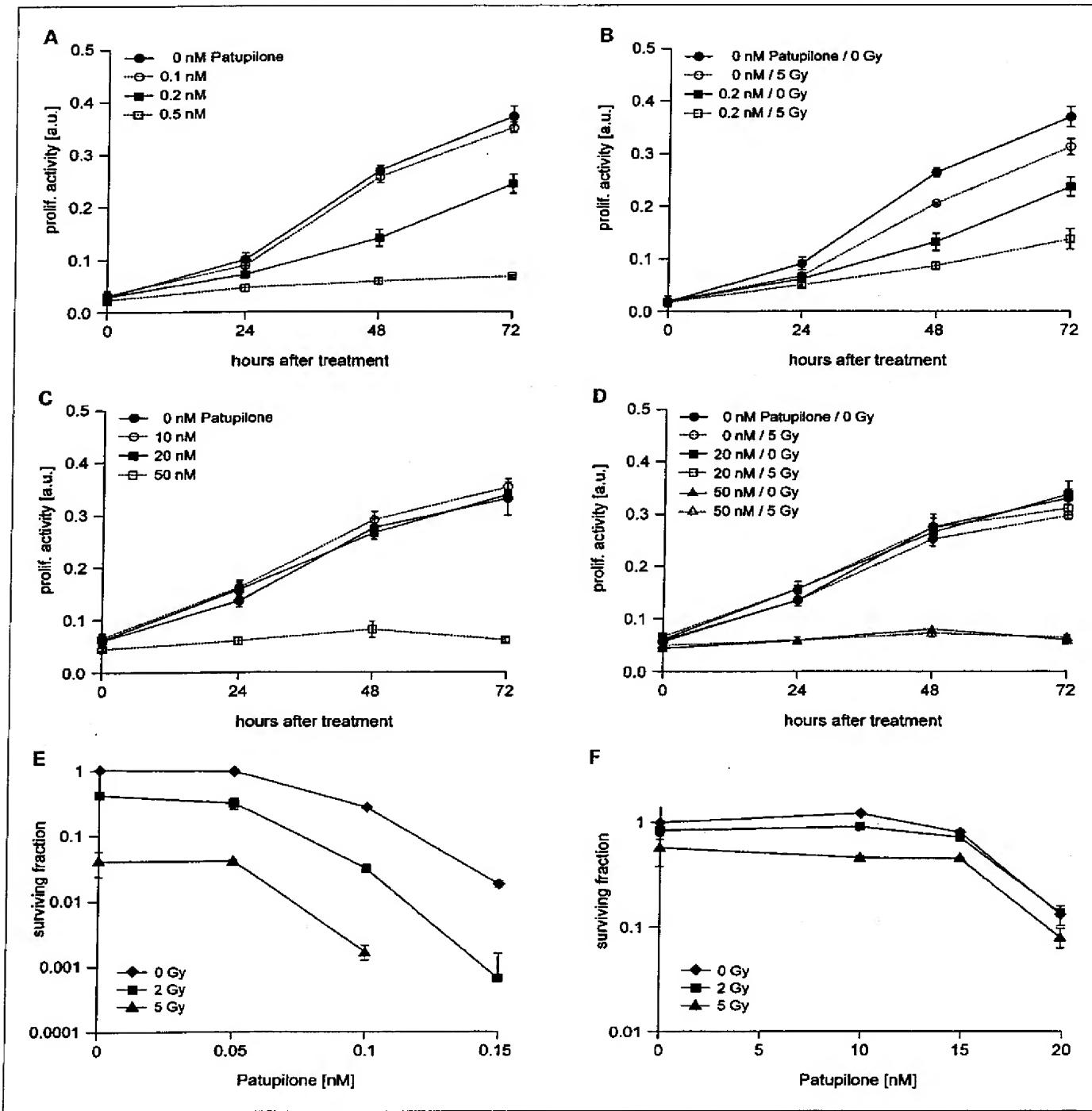


Fig. 2. Antiproliferative effect of patupilone alone and in combination with IR (5 Gy). Lung adenocarcinoma cells A549 (**A** and **B**) and mutant cells A549.EpoB40 (**C** and **D**) were exposed to increasing concentrations of patupilone and IR. Clonogenic survival after treatment with patupilone and IR was determined for A549 cells (**E**) and mutant A549.EpoB40 cells (**F**) treated with patupilone 18 h before irradiation.

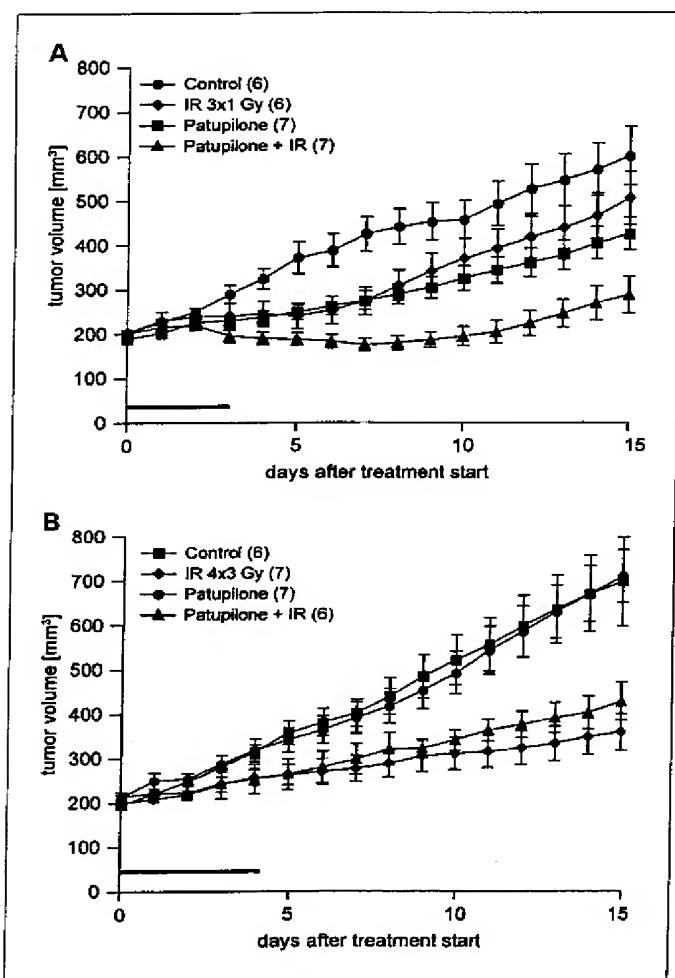


Fig. 3. Effect of patupilone and IR alone or in combination on the growth of A549-derived (**A**) and A549.EpoB40-derived (**B**) xenografts in nude mice. Mice were treated with patupilone (2 mg/kg once) and IR (3 × 1 or 4 × 3 Gy on consecutive days) alone and in combination, with administration of patupilone or the vehicle 24 h before the first fraction of IR. Horizontal column, days of treatment. Mean ± SE tumor volume per group.

initiation. No skin changes or tissue damage were observed in the coirradiated healthy tissue area around the tumor.

Histologic analysis of treatment-induced effects in tumor xenografts. To investigate a putative antiangiogenic effect of the different treatment modalities, MVD, tumor hypoxia, and proliferative status were determined in tumor sections of treated mice, which were collected 84 h after treatment with patupilone (2 mg/kg) or vehicle alone, after fractionated irradiation (3 × 1 or 3 × 3 Gy, respectively) or the combined treatment modality. Histologic analysis was first done on xenografts without any treatment to compare baseline characteristics of A549 and A549.EpoB40 xenografts. Xenografts of both A549 and A549.EpoB40 cells showed solid growth of carcinoma cells with areas of necrosis and thin stromal septa.

Tumor cell proliferation was determined using immunohistochemistry for the Ki-67 protein, which is expressed during all phases of the cell cycle, except G₀. In the tumors derived from the drug-sensitive A549 cell line, the proliferative activity was

reduced by 32% 84 h after treatment with patupilone alone, whereas irradiation and the combined treatment reduced the proliferative activity by 54% and 71%, respectively ($P < 0.0001$). No change in the proliferative activity could be detected in A549.EpoB40-derived tumor sections after patupilone treatment. Irradiation reduced the proliferative activity by 22% ($P < 0.0001$), without any further effect in the combined treatment group (Fig. 4A and B). To examine the effect of the different treatment modalities on the tumor vasculature, MVD was determined by CD31 immunohistochemistry. Patupilone reduced the MVD in the drug-sensitive tumors by 25% at the 84 h time point when compared with control tumors ($P = 0.0003$). Irradiation and combined treatment reduced the MVD by 37% and 47%, respectively ($P < 0.0001$). In the drug-resistant tumors, a tendency in MVD reduction was only observed in the irradiated group ($P = 0.074$; Fig. 4C and D).

Treatment-induced changes of the tumor vasculature may also affect the pO₂ in the tumor tissue. We therefore determined treatment-dependent changes of tumor oxygenation by injection of the hypoxia probe pimonidazole, which specifically accumulates in hypoxic tissue areas. Treatment-dependent changes of hypoxic areas were determined in histologic sections 84 h after treatment. A significant increase of pimonidazole accumulation was determined in A549-derived tumors after treatment with patupilone ($P = 0.0019$), whereas the amount of hypoxic, pimonidazole-positive areas was reduced in the irradiated group ($P = 0.0045$). Interestingly, the amount of hypoxic areas was similar to untreated tumors after treatment with patupilone in combination with IR ($P = 0.747$). No change of pimonidazole accumulation was observed in any of the treatment groups in A549.EpoB40-derived tumor sections (Fig. 4E and F). These results indicate that changes in the tumor microenvironment in response to the different treatment modalities mainly derive from the treatment effect directed against the tumor cells (see below).

Treatment-interfered expression of hypoxia-induced gene expression. The HIF represents the major determinant in tumor cells for hypoxia-regulated expression of proangiogenic factors and patupilone reduced the level of the hypoxia-sensitive HIF-1 α subunit under hypoxic conditions in the patupilone-sensitive A549 cell line as determined by Western blotting (data not shown). Using a reporter gene assay where luciferase expression is under the control of multiple HREs, the treatment response of patupilone and IR alone and in combination was quantitatively assessed in the patupilone-sensitive and patupilone-resistant cell lines. To normalize for transfection efficacy and cell numbers, cells were cotransfected with the Renilla luciferase-expressing vector system. To mimic hypoxic conditions, cells were incubated in dimethyloxaloylglycine, thereby up-regulating the endogenous HIF level and inducing luciferase expression 8.5-fold over control conditions (data not shown). Treatment of the patupilone-sensitive A549 cells with increasing doses of the MSA significantly reduced luciferase expression already at subnanomolar concentrations ($P = 0.010$). Of note, the A549.EpoB40 cells were resistant up to low nanomolar concentrations of patupilone (20 nmol/L), indicating a role of microtubule interference in HIF-1 α -regulated gene expression (Fig. 5A). Treatment of cells with increasing doses of irradiation only minimally affected luciferase expression (data not shown). Treatment with increasing doses of patupilone in combination with IR (5 Gy) additionally

decreased luciferase expression ($P = 0.018$) in the A549 cell line, indicating a increased stress response on the level of hypoxia-regulated gene expression (Fig. 5B).

Discussion

Here, we investigated the treatment response of tumor xenografts derived from MSA-sensitive and MSA-resistant lung

adenocarcinoma cell lines to treatment with patupilone and IR alone or in combination. We previously identified a strong treatment response to this combined treatment modality *in vivo* in the SW480 human colon xenograft model, which strongly exceeded the additive cytotoxic effect of the treatment combination *in vitro* (15). By means of this genetically defined tumor model, we now were able to differentiate between a direct tumor cell-directed and a microenvironment-directed

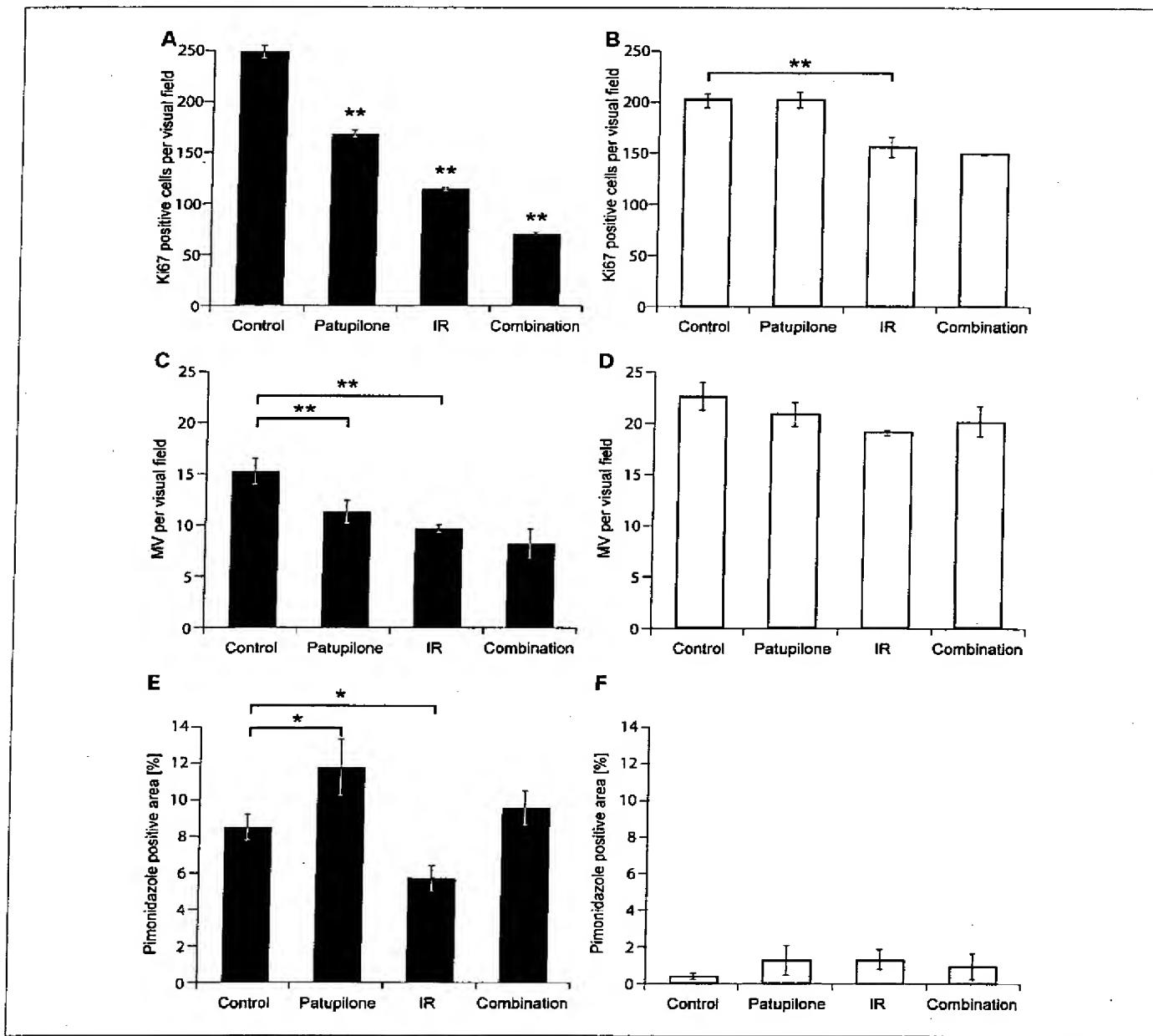


Fig. 4. Tumor cell proliferation, MVD, and changes in tumor hypoxia in response to treatment. Mice with A549-derived (A, C, and E) and A549.Epo840-derived (B, D, and F) xenografts were treated with vehicle, patupilone (2 mg/kg), IR (3 × 1 and 3 × 3 Gy, respectively), or a combination of patupilone and IR. Mice were sacrificed 84 h after treatment and tumors were harvested, formalin fixed, and stained for the Ki-67 (A and B), CD31 as marker of tumor cell proliferation and MVD (C and D), and antibodies against the exogenous hypoxia marker pimonidazole hydrochloride for the immunohistochemical detection of tumor hypoxia (E and F), respectively. Mean ± SE value per group. *, $P < 0.05$; **, $P < 0.001$.

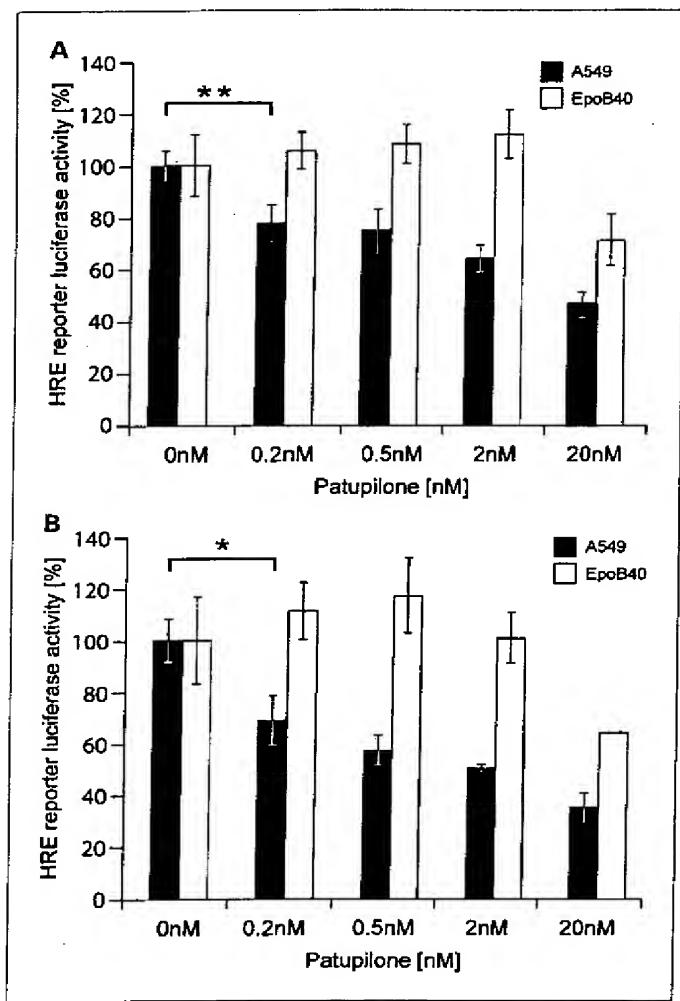


Fig. 5. Patupilone reduces HRE-dependent luciferase expression. A549 and A549.EpoB40 cells stably transfected with a pGL4.27 HRE vector (3× HRE-VEGF promoter for firefly-luciferase expression) and the pRL SV40 vector (*Renilla* luciferase) were treated with increasing doses of patupilone (A) and in combination with IR (5 Gy; B) under normal and hypoxia mimicking conditions. The cells were tested for luciferase reporter activity 24 h after patupilone treatment. Mean ± SE value per group. *, P < 0.01; **, P < 0.001.

cytotoxic *in vivo* effect in response to the different treatment modalities.

We observed a cytotoxic and tumor growth delay effect in the tumors derived from the MSA-sensitive adenocarcinoma cell line A549 in response to irradiation and patupilone alone and a supra-additive response to combined treatment. As expected, patupilone reduced the proliferative activity of the MSA-resistant cell line A549.EpoB40 *in vitro* only at >100-fold the concentration necessary for the wild-type cell line (3, 18). As determined here in this report, no tumor growth delay could be observed *in vivo* with treatment of patupilone alone and no enhancement of an IR effect was induced on combined treatment in tumors derived from the MSA-resistant cell line. Of note, patupilone induced an antiangiogenic response with reduced MVD and enhanced hypoxia in the wild-type but not MSA-resistant tumors.

The wild-type A549 cell line was also more sensitive to treatment with increasing doses of IR than A549.EpoB40 cells, which carry a specific Gln-to-Glu mutation at residue 292 in the M loop of β-tubulin and which changes the affinity of β-tubulin to the MSA (3). We cannot exclude that additional mutations are present in the patupilone-resistant cells, but most likely the known β-tubulin mutation not only reduces the affinity to patupilone but also affects microtubule dynamics, which may subsequently render the cells more radiation resistant. Importantly, the proliferative activity and growth kinetics of untreated cells or tumors derived from the two different cell lines were similar. An increased radiation resistance was present in the patupilone-resistant tumor cells and not in the sensitive ones and thus still enabled to investigate the involvement of the tumor microenvironment in response to the combined treatment modality of IR with patupilone.

The potent antiangiogenic activity of MSAs was proposed to be a major mechanism leading to the successful anticancer activity of this class of compounds (9, 11, 19, 20). Patupilone has an antiproliferative and apoptosis-inducing effect in microvascular and macrovascular endothelial cells (9) and our own *in vitro* experiments done with primary human umbilical vein endothelial cells confirmed the high potency of patupilone against this cell type (data not shown). Patupilone also induces vascular disruption leading to reduced tumor blood volume (8). We showed previously an at least additive antitumor effect by the combined treatment of IR with different inhibitors of angiogenesis, such as the VEGF receptor tyrosine kinase inhibitor PTK787 or the dual receptor tyrosine kinase inhibitor AEE788 (21, 22). Based on the putative direct antiangiogenic property of patupilone, a partial tumor growth delay effect was expected on treatment with patupilone alone or in combination with IR even in tumors derived from the patupilone-resistant tumor cells (A549.Epo40). However, treatment with neither patupilone alone nor in combination with IR reduced tumor growth or increased tumor growth delay, respectively, in A549.Epo40-derived tumors.

A strong reduction in MVD and change in tumor hypoxia on treatment with patupilone alone was only detected in tumors derived from the A549 wild-type cells and not in tumors derived from the patupilone-resistant tumor cells. Despite direct *in vitro* cytotoxicity against endothelial cells (9), our results indicate that the antiangiogenic effect of patupilone *in vivo* is indirectly induced by interference on the level of the tumor cellular stress response. Our *in vitro* experiments done with the hypoxia-regulated reporter gene assay clearly showed that patupilone reduces the expression of the HIF-1 "transcriptome" such as VEGF and other genes involved in angiogenesis and hypoxic adaptation but only in patupilone-sensitive tumor cells, thereby leading to an indirect tumor cell-mediated antiangiogenic effect of patupilone. Patupilone might also directly target the microtubule system in the tumor endothelial cell compartment but might be less cytotoxic in presence of sufficient VEGF and other survival factors secreted from the tumor cells. The radiosensitivity of the tumor vascular network codetermines the tumor response to IR (23). Therefore, pharmacologic approaches, which interfere with the survival signaling of endothelial cells, render these cells more radiosensitive and subsequently enhance the tumor response to IR.

A major obstacle for the successful tumor response to IR is tumor hypoxia, which renders tumor cells two or three times more radioresistant than under normoxic conditions. Of note, our *in vitro* experiments revealed that patupilone is as potent against tumor cells under normoxic as under hypoxic conditions. Thus, a combined treatment modality of patupilone in combination with IR also bears promise against tumors with an increased hypoxic fraction. Here, we observed an increase of tumor hypoxia induced by patupilone alone presumably through this antiangiogenic, indirect MVD-reducing effect. We investigated previously the dynamics of tumor hypoxia in response to inhibitors of angiogenesis alone and in combination with IR and observed that irradiation counteracts the risk of treatment-induced hypoxia by the inhibition of angiogenesis (21, 22). Although treatment with the VEGF receptor/tirosine kinase inhibitor PTK787/ZK222584 also increased overall and local tumor hypoxia, combined treatment with IR resulted in extended tumor growth delay and tumor cell apoptosis but no increase in tumor hypoxia (22). As the radiosensitivity of the tumor vascular network codetermines the tumor response to IR (23), pharmacologic approaches, which interfere with the survival signaling of endothelial cells, render these cells more radiosensitive and subsequently enhance the tumor response to IR. The reduction of survival factors secreted by the tumor cells, after treatment with patupilone, leaves the endothelial cells more vulnerable to direct irradiation. Nevertheless, combined treatment of patupilone with radiation did not enhance tumor hypoxia relative to untreated tumors as if radiation antagonizes the patupilone-induced increase of tumor hypoxia. The mechanisms underlying this antagonistic effect is unclear, but

it is conceivable that enhanced cell death together with reduced proliferation of tumor cells may reduce the intratumoral oxygen demand to a level that can still be met by the damaged tumor vasculature and thereby avoid a hypoxic state in the allograft (24, 25). This mechanism might be relevant for the combined treatment of irradiation with direct and indirect inhibitors of angiogenesis.

In summary, our results using *in vivo* tumor models derived from patupilone-sensitive and patupilone-resistant variants of the non-small cell lung cancer cell line A549 strongly suggest that the major antitumor effect of patupilone and a combined patupilone/IR treatment is mainly due to directed effects on the tumor cell compartment and that patupilone may induce an antiangiogenic effect in an indirect way through tumor cell targeting. Overall, these preclinical experiments are of strong relevance for a clinical strategy and further support the promising treatment modality of patupilone alone and in combination with IR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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